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Surface analyses of biocements from *Pectinaria gouldii* (Polychaeta: Pectinariidae) and *Phragmatopoma lapidosa* (Polychaeta: Sabellariidae)

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Abstract

Pectinaria gouldii and *Phragmatopoma lapidosa* are marine polychaetes that reside in protective structures built from sand grains bound together using proteinaceous cement secreted from specialized glands. *P. gouldii* constructs a solitary, ice-cream-cone-like structure. The smaller, gregarious *P. lapidosa* forms a large, reef-like mound. This study investigates the physical features of these two polychaete biocements, linking structure and function in two marine environments.

The surface structures of hydrated biocement samples were analyzed using atomic force microscopy (AFM), and the surface structures and composition of dehydrated biocement samples were analyzed using scanning electron microscopy (SEM) and electron dispersive spectroscopy (EDS). Atomic force analyses indicate that (in their native states) the surface roughness, adhesion, and stiffness of *P. gouldii* biocement are greater than *P. lapidosa* biocement. The surface of *P. gouldii* resembled "cottage cheese," while the surface of *P. lapidosa* had smoother features. SEM revealed "popped bubble" features that indicated a solid foam-like material for both biocements. EDS confirmed the presence of calcium, magnesium, and phosphorous in both biocements, with varying amounts of these three elements at different locations on the same sample.

Key words: AFM, EDS, SEM, SPM, force curves, cement, surface roughness, solid foam

Introduction

Pectinaria gouldii (Verrill) and *Phragmatopoma lapidosa* (Kinberg) are small polychaetous annelids found in intertidal and subtidal regions along the Atlantic coastline. The benthic, solitary living *P. gouldii* is also known as a trumpet worm, or ice cream cone worm and can grow to be up to 5 cm in length (Remsen 2007). It uses sand grains to construct its tube, which has an ice cream cone-like structure that is open at both ends. Sand grains are selected by size, then fitted and cemented together in a single layer. The smaller, gregarious *P. lapidosa* form reef-like mounds that can vary in size from a softball to large boulder-like formations that can be part of a network that stretches for miles (Kirtley & Tanner 1968; U.S. Department of the Interior Fish and Wildlife Service 1989). These large structures form reefs that help shelter beaches by absorbing the force of the waves (U.S. Department of the Interior Fish and Wildlife Service 1989). Both polychaetes utilize a type of cement that is secreted from specialized glands to attach sand particles together in order to build their

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dwellings. Some fascinating properties of these biocements are their ability to harden in seawater and withstand ocean waves, tide cycles, and other forces prevalent along the coast. This study extends the knowledge and understanding of the physical features of these two polychaete biocements, linking structure and function in two marine environments.

In this report, we use atomic force microscopy (AFM) and scanning electron microscopy (SEM) to investigate the similarities and differences that exist in topology and general surface features of the biocements produced by two polychaetes, *Pectinaria gouldii* and *Phragmatopoma lapidosa*. By using AFM, we compare the relative roughness, adhesion, and stiffness of the samples (Tsui et al. 2000; Salerno & Bykov 2006). Energy dispersive spectroscopy (EDS) data provides elemental comparison in the composition of the biocements.

AFM is one type of scanning probe microscopy (SPM). It uses a cantilever that has a tip that comes into contact with the sample surface. As the tip is in contact with the sample, surface interactions are measured by a photodetector recording a beam being reflected off the backside of the cantilever (Radmacher et al. 1992). AFM allows topographical surface investigation of the samples in their native, hydrated state. It is this ability to analyze topography that makes AFM an ideal instrument in this study. SEM utilizes an electron beam striking the sample and the emitted secondary electrons are detected to form an image. Sample preparations of SEM require dehydration which alters the native structure of the biocements. Desiccated cement samples are more brittle and weak as compared to their hydrated states. These two methods, AFM and SEM, allow topographical analyses on the nanometer scale. When EDS is done with SEM, X-rays emitted from the sample allow elemental analyses since the X-ray energies correspond to individual elements. All of these techniques were used in this report to investigate the biological cement secreted by *Pectinaria gouldii* and *Phragmatopoma lapidosa.*

Materials and methods

Colonies of *Phragmatopoma lapidosa* were collected from Ft. Walton Beach, FL, and maintained in an Instant Ocean (Aquarium Systems Inc.) saltwater tank at ambient temperature. They were fed live phytoplankton (DT's Plankton Farm). *Pectinaria gouldii* samples were ordered from Marine Biological Laboratory, Woods Hole, MA, and were maintained under the same conditions as *P. lapidosa*.

In order to acquire suitable cement samples of *Phragmatopoma lapidosa*, individual worms were removed from the reef with their tubes intact and placed on a bed of glass beads that measured 0.1 mm, 0.5 mm, or a combination of sizes (Biospec Products, Inc.) using the method of Jensen & Morse (1988). Most of the organisms repaired and extended their tubes with the surrounding glass beads. The rebuilt sections of tubes that were made from these beads were removed for analysis. The advantage of this approach was twofold: the sample was easier to manipulate in the AFM due to consistent bead size, and it was easier to see the biocement against the transparent glass. Trying similar tactics to stimulate *Pectinaria gouldii* to make new cement to reconstruct their tube has proven unsuccessful. The samples of *P. gouldii* were prepared by removing sections of the tubes with forceps.

A Veeco-multimode SPM Nanoscope IV was the AFM instrument used for analysis of relative roughness. All samples were mounted on aluminum pucks and kept hydrated with saltwater (Instant Ocean). The AFM was operated under tapping mode with a standard silicon cantilever (Veeco, RTESP7); its resonant frequency ranged from 281 to 331 kHz. Scan sizes for *Phragmatopoma lapidosa* ranged from 5–8 µm square, most were 5 µm. *Pectinaria gouldii* scans ranged in size from

5–20 µm square, with 10 µm being the most frequent. Data analysis was completed using Gwyddion SPM analysis software (version 2.7) to obtain roughness values. All the images presented are the original data captured by the Veeco AFM. Scans taken with this instrument were done at Coe College, Cedar Rapids, IA.

An Asylum MFP-3D SA AFM was used for force modulation. The tips were CSC37/AlBS and CSC17/AlBS (MikroMasch). All data were taken while the sample was submerged in salt water. All force modulation data analysis was done with Igor Pro 5.05A with the MFP-3D suite installed (3D050811+610). Scans taken with this instrument were done at University of Iowa, Central Microscopy Research Facility, Iowa City, IA.

A Hitachi S-4000 SEM was used for structural analyses. The cement samples were prepared by rinsing with deionized water, air drying, and desiccating under a vacuum. Once dry, the samples were mounted onto a SEM stage by attachment with carbon tape and carbon sputter coated. Both *Phragmatopoma lapidosa* and *Pectinaria gouldii* cement samples were prepared in the same way. A Hitachi S-3400N SEM with a Bruker AXS signal processing unit was used to obtain EDS information. Samples that underwent EDS were prepared as previously described, but were not carbon coated. Scans taken with this instrument were done at University of Iowa, Central Microscopy Research Facility, Iowa City, IA.

Results and discussion

Gibson and Ashby (1997) describe a cellular solid as one made up of an interconnected network of bubble like structures, i.e., a honeycomb. The properties of cellular solids are advantageous to the dwellings of *Pectinaria gouldii* and *Phragmatopoma lapidosa*. One of these properties is the ability to absorb the energy of impacts, which allows *P. lapidosa* to handle wave action along the Atlantic coastline and allows *P. gouldii* to maintain structural integrity under benthic conditions. A second property may be reliable thermal insulation which helps them to tolerate temperature fluctuations of the ocean environment. An artificial cellular solid, Styrofoam, is modeled from a natural cellular solid to exploit these same two features. We feel that both properties allow the biocement structures to efficiently handle an often stressful environment.

Viscous forces in liquid can have a profound effect on topology. The biocement, a solid foam, is formed in sea water, and some water is likely incorporated into its bubble-like compartments. Like most natural foams, both biocements are anisotropic, which occurs as these compartments elongate in a given direction. A variation in foam bubble compartment size for *Phragmatopoma lapidosa* (0.1–5 µm) was seen consistently in SEM scans. *Pectinaria gouldii* had foam bubble compartment sizes in the range of 5–7 µm. Gibson and Ashby (1997) give multiple examples of how structural and material anisotropy combine to maximize the structural efficiency of both biocements.

Over a dozen scans of each type of biocement were analyzed by SEM. The commonly seen solid foam nature of *Pectinaria gouldii* and *Phragmatopoma lapidosa* biocement is viewed in Figs. 1 A and C. *P. gouldii* forms a more uniform solid foam with bubble compartment sizes of approximately 5–7 µm. *P. lapidosa* exhibits more variation in bubble compartment size, approximately 0.1–5 µm. In addition to the foam structure, *P. lapidosa* presents a fibrous attachment between glass beads in Fig. 1 D. The analyses of the biocements by SEM were vital to the study of the physical features. It appears that the lumpy characteristics seen in AFM (Figs. 3 A, D) are due to the bubbly features in SEM (Figs. 1 A, C) that show the solid foam compartments. Even though there is a disadvantage in examining the cement in a dehydrated state, SEM provided an insightful view of the physical nature of the cement.

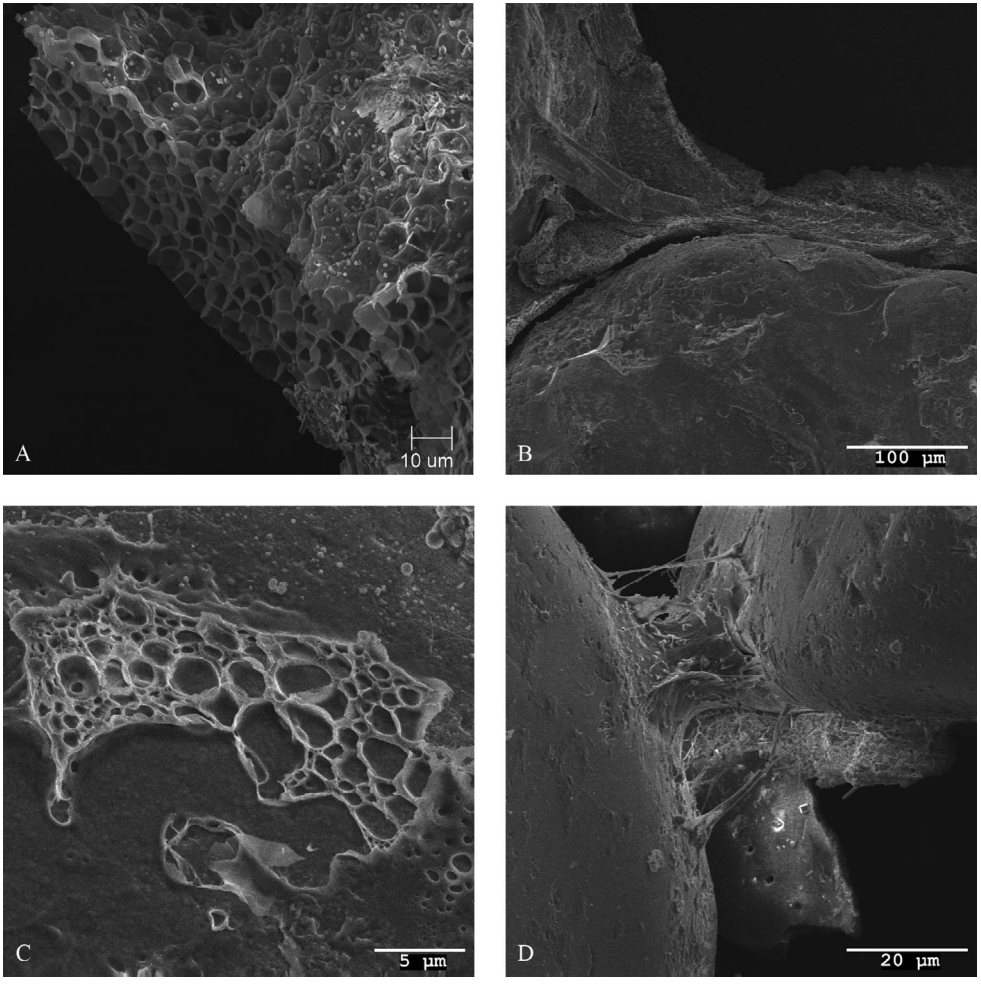

FIGURE 1. SEM images of biocements in dehydrated states. A, *Pectinaria gouldii* cement with uniform cellular solid foam features, 4–6 µm cell size; B, *P. gouldii* cement shown at the interface of a sand granule; C, *Phragmatopoma lapidosa* cement with variable cellular foam features, 0.1–3 µm cell size; D, *P. lapidosa* cement with fibrous and foam features shown between two glass beads.

The primary advantage of the AFM analyses is the ability to measure surface features of the biocements in their native, hydrated states. In Fig. 2, the AFM tip is shown positioned on an edge of a cement region ready for scanning. By comparison, the cement regions available for analysis were larger in *Pectinaria gouldii* samples than in *Phragmatopoma lapidosa* samples. Although there were some individual variations, the same basic features were prevalent in all of the samples scanned. Smooth areas of cement were typically found along its contact edges with the sand grain or glass bead. The cleft regions were usually found near the center of the cement regions. These clefts may reflect a depression in the fresh deposition of the soft cement that results from the pressure of the attachment of a new sand grain or glass bead. Figs. 3 A and D illustrate the lumpy features of the

biocements. *P. gouldii* cement has larger surface features ranging from 2–5 µm, while *P. lapidosa* features were usually less than 2 µm. This consistent topology was seen in all samples. The lumpy features (Figs. 3 A, D) and clefts (Figs. 3 B, C) of both biocements were reflected in the roughness data (see below). The bubbly nature of both biocements was evident in SEM and supported previous SEM analyses in related organisms, *Phragmatopoma californica* (Stewart *et al*. 2004) and *Pectinaria kornei* (Truchet & Vovelle 1977; Volvelle 1979).

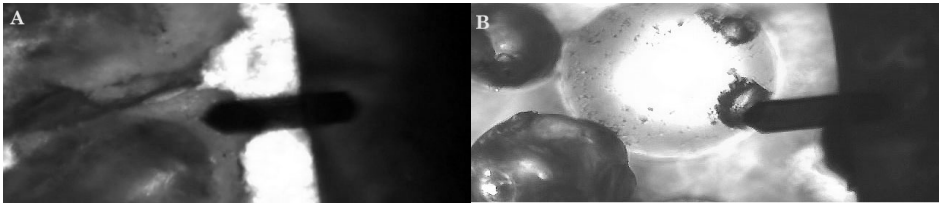

FIGURE 2. A, AFM tip positioned over an area of cement for a *Pectinaria gouldii* sample (10X magnification); B, AFM tip positioned over an area of cement attached to a glass bead for a *Phragmatopoma lapidosa* sample (10X magnification).

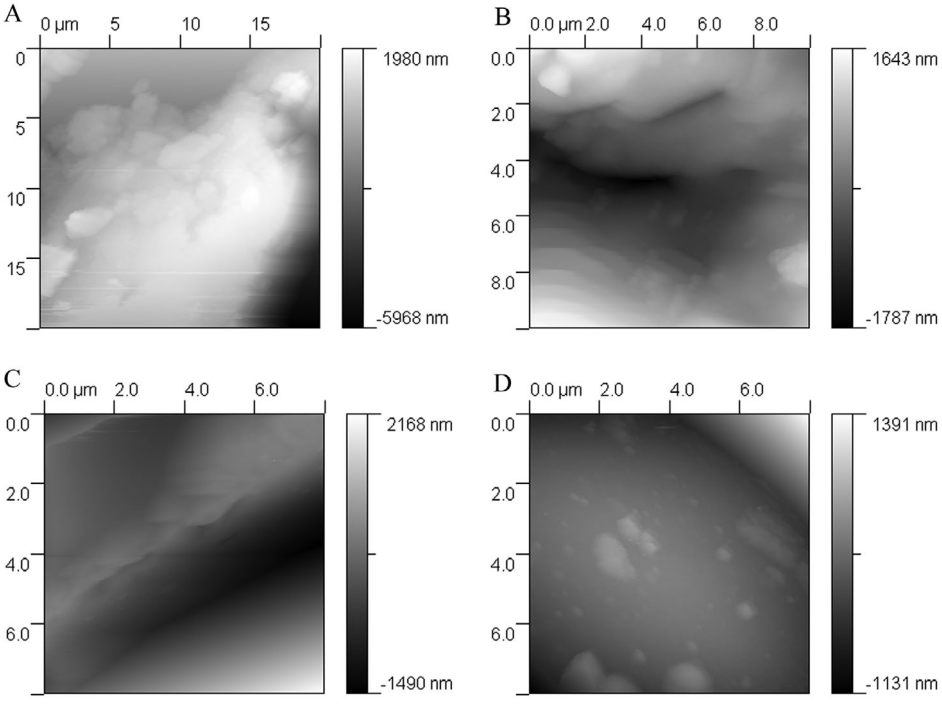

FIGURE 3. AFM topographic images of biocement in hydrated, native state. A, *Pectinaria gouldii* sample showing ~2–5 µm diameter "lumpy" surface features, 20 µm scan; B, *P. gouldii* sample showing cleft and "lumpy" surface features, 10 µm scan; C, *Phragmatopoma lapidosa* cleft feature, 8 µm scan; D, *P. lapidosa* showing "lumpy" surface features less than 2 µm diameter, 8 µm scan.

We collected 22 scans of *Phragmatopoma lapidosa* and 21 scans of *Pectinaria gouldii* using AFM in tapping mode. By doing RMS roughness analysis with Gwyddion on the collected scans and comparing *P. gouldii* to *P. lapidosa*, it was shown that *P. gouldii* cement was 2.3 times rougher than *P. lapidosa*. The difference in biocements is attributed to topological features: the number of lumps versus smoothness versus clefts. An effort was made to analyze a variety of cement samples so the results would not be biased toward any type from a particular region. Small pieces of diatoms, shells, and other debris hindered the analyses of several samples of *P. gouldii*. The AFM tip would adhere to the debris and cause discontinuity in the scans. Debris deposited by wave action on the surface of *P. lapidosa* cement interfered with direct scanning of reef samples. Because *P. lapidosa* were allowed to rebuild tubes using glass beads in a saltwater tank, the debris problem was eliminated and the features of the cement could be measured directly.

By using the Asylum AFM, we were able to obtain 91 force curves for *Pectinaria gouldii* and 152 forces curves for *Phragmatopoma lapidosa* (Fig. 4). A force curve is a plot of the forces acting on the cantilever as it approaches and retracts from the surface of a sample (Almqvist et al. 2004). These force curves were obtained in two different data sets with approximately an equal number of force curves each time. The force curves were taken in fluid, which eliminated any capillary forces present and allowed us to see specific interactions between the tip and the sample (Radmacher et al. 1994). From these force curves we compared adhesion and relative stiffness for each sample. By comparing the slope of these force curves we could determine the relative stiffness of each of the samples. Softer samples indent more readily, which requires more applied force to gain the same amount of deflection as would be produced by a harder sample (Radmacher et al. 1995; Laney et al. 1997; Support note 1999). Since we ran force modulation with two different cantilevers that had drastically different spring constants, comparing actual values of slopes obtained from the force curves may be misleading. Instead, we compared the slopes between *P. gouldii* and *P. lapidosa* within each of the data sets and were able to see which sample was more flexible. We found that *P. gouldii* biocement is stiffer than *P. lapidosa* biocement. This was consistently seen throughout each of the data sets. We hypothesize that since *P. lapidosa* is more exposed to wave action, its cement evolved to be more flexible. If the cement was stiffer, it would be more likely to rupture in the wave action (Stewart et al. 2004).

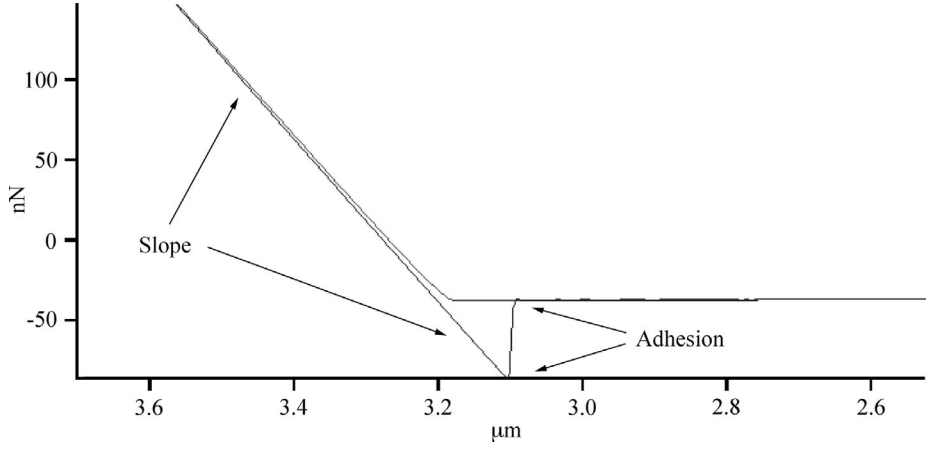

FIGURE 4. Force modulation curve with "stiffness" indicated by the slope and "adhesion" values from a representative *Phragmatopoma lapidosa* sample.

Adhesion data was also extracted from the collected force curves. This was done by measuring the maximum amount of force before the tip releases from the sample surface (Tsui et al. 2000; Salerno & Bykov 2006). *Pectinaria gouldii* had approximately the same adhesion for both sets of data that we acquired (Table 1). Interestingly, *Phragmatopoma lapidosa* had two different adhesion values for each set. This was attributed to a curing time that appears to be present in the cement. The first time we acquired data the cement was 3 days old, whereas the second time it was 10 days old. A change in cement appearance was noted in a similar worm, *Phragmatopoma californica* (Stewart et al. 2004). A curing time may be present in *P. gouldii*, but we were unable to test this due to the difficulty of stimulating the adult worms to repair or extend preexisting cone structures, as previously mentioned.

TABLE 1. AFM average adhesion values in nanoNewtons (nN) for *Pectinaria gouldii* and *Phragmatopoma lapidosa*.

	***P. gouldii* (nN)**	***P. lapidosa* (nN)**
First data set	4.56	10.2
Second data set	4.92	2.04

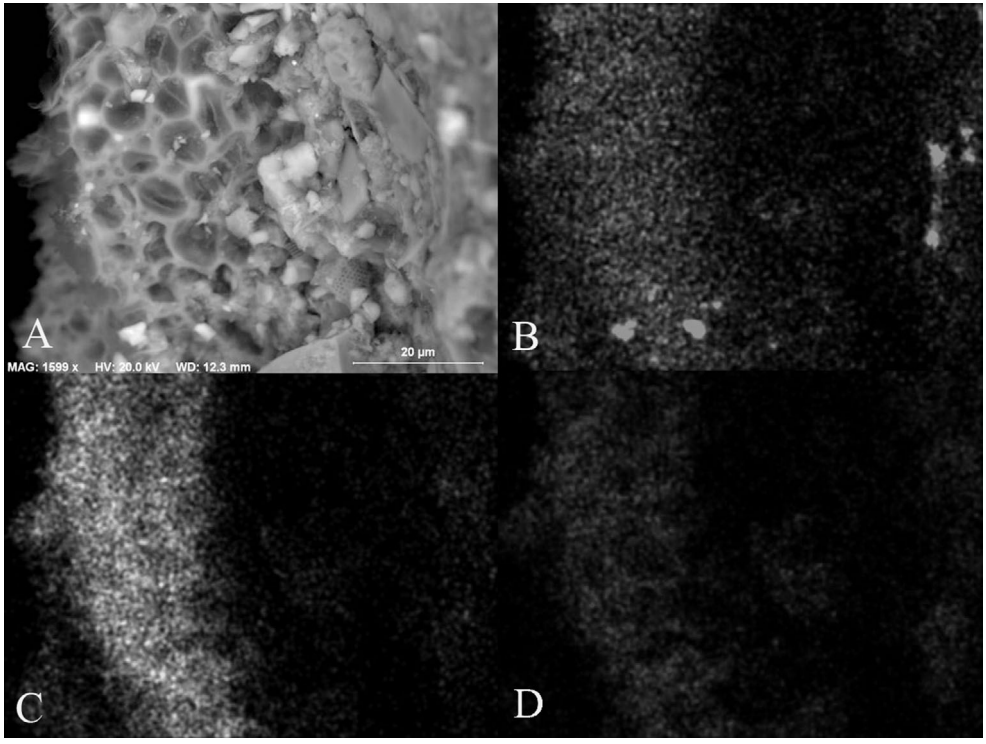

FIGURE 5. *Pectinaria gouldii* biocement. A, SEM scan for reference; EDS scans showing (B) calcium, (C) phosphorus, and (D) magnesium.

The information obtained by EDS mapping was particularly useful in analyzing the differences in elemental composition between the two biocements (Figs. 5–6). Both biocements present high levels of magnesium and phosphorus, indicating similarity in the composition between the two biocements, but the differences in calcium levels suggest that the biocements accomplish their function by slightly different mechanisms. Metal ions are commonly used to help crosslink proteins noncovalently. The high levels of phosphorus in both biocements correlate well with already published data on the sequence of a suspected *Pectinaria gouldii* cement protein, which shows high levels of serine (Briggs et al. 2004). Serine is a commonly phosphorylated amino acid, therefore when high levels of serine are seen, high levels of phosphorus can be expected. Additionally, *Phragmatopoma californica* has already been reported to also have high levels of serine associated with cement proteins (Zhao et al. 2005).

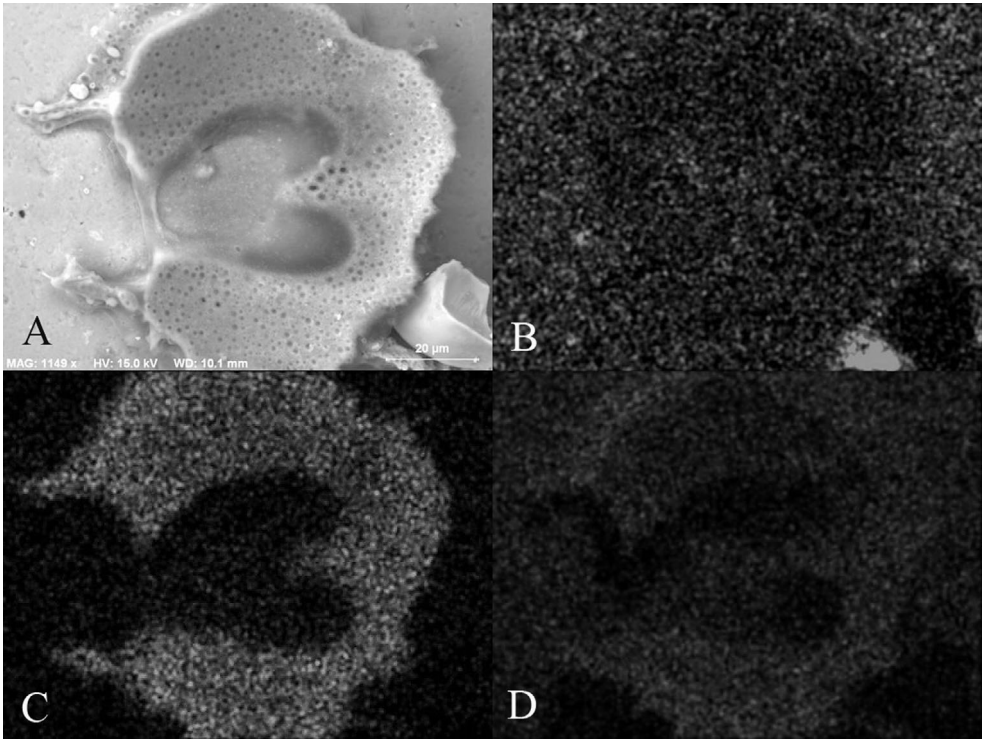

FIGURE 6. *Phragmatopoma lapidosa* biocement. A, SEM scan for reference; EDS scans showing (B) calcium, (C) phosphorus, and (D) magnesium.

The combination of AFM, SEM, and EDS spectroscopies provided a unique combination of tools to study marine biocements in their native and dehydrated states. *Pectinaria gouldii* biocement is relatively rougher and stiffer than *Phragmatopoma lapidosa* biocement, which seems to mirror the environmental needs of the worms in their respective environments. Curing changed only the adhesion values for *P. lapidosa* biocement, but not its stiffness. It appears that the solid foam nature of the biocements contributes to their environmental stability. Both biocements contain magnesium, calcium, and phosphorus, although calcium seems to be more plentiful in *P. gouldii* biocement.

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